J. Enzyme Inhibition, 1993, Vol. 7, pp. 77–85
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PEPTIDYL AMMONIUM METHYL KETONES AS SUBSTRATE ANALOG INHIBITORS OF PROLINE-SPECIFIC PEPTIDASES

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(Received 2 February 1993)

Prolyl endopeptidase (PEP) and dipeptidyl peptidase IV (DP IV) are serine enzymes cleaving highly specific prolyl peptide bonds. Both enzymes were found to be inhibited by newly designed peptidyl ammonium and pyridinium methyl ketones acting as slow binding inhibitors. The most potent inhibitor of PEP is Z-Pro-Pro-$CH_2N^+C_5H_5$ exhibiting a K_i^* value of 1.8 nM with a first-order rate constant of k_{on} 0.0022 s^{-1} for the formation of the tight enzyme-inhibitor complex. DP IV and H-Pro-Pro-$CH_2N^+(CH_3)_3$ form an enzyme-inhibitor-complex with an apparent second order rate constant of 2713 $M^{-1}s^{-1}$. In contrast to the very stable N-terminal protected Z-Pro-Pro-$CH_2N^+(CH_3)_3$, the deblocked derivative decomposes rapidly in aqueous solution.

KEY WORDS: Prolyl endopeptidase, dipeptidyl peptidase IV, inhibition, methylketones.

INTRODUCTION

Many regulatory peptides (i.e. neuropeptides, immunopeptides, peptide hormones) share the common feature of having proline residues at positions in their sequence where they fulfill two main tasks:[1] determining the properties of the secondary structures of the peptides, necessary for their biological activity (e.g., membrane passage, receptor binding) and serving as special cleavage points for proline-specific peptidases, which are widely believed to be the converting enzymes of those peptides.[1] Physiological processes of protein folding and signal transmission as well as undesirable functions like HIV-maturation caused by -X-Pro- specific aspartate protease are related to the mentioned properties of proline. Work dealing with mechanistic features of the proline-specific enzymes including inhibitor design seems therefore of general interest.[2–4]

Among these enzymes, prolyl endopeptidase (PEP) and dipeptidyl peptidase IV (DP IV) exhibit certain similarities in their catalytic behavior, i.e., similar rate constants

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for substrate hydrolysis, preference of proline over alanine in P_1-position of similar substrates and drastic discrimination of other amino acids at the cleavage site.[5] Recent studies suggest that PEP is an enzyme involved in learning and memory process[6] and it was shown to be responsible for the generation of the amyloid A4-peptide in Alzheimer's disease.[7,8] DP IV seems to be involved in signal transmission during the immune response. The T cell triggering molecule Tp103 was demonstrated to be this enzyme.[9,10] Our studies on mechanism-based protease inhibitors show that in contrast to other serine and cysteine proteases which are inactivated efficiently by *N*-peptidyl-*O*-acyl hydroxylamines, PEP and DP IV mainly hydrolyze these compounds as substrates,[11,14–16] pointing towards a different catalytic mechanism of the proline-specific serine enzymes.

Recently, proline-containing peptide aldehydes and boronic acids have been tested as inhibitors of the proline-specific peptidases, but their instability due to the reactive electrophile should limit their potential use in biological systems.[12,13] However, acyl pyrrolidides and several deduced derivatives have been found to be powerful and stable reversible inhibitors of this enzymes, inhibiting the target proteins in the μM to nM-concentration range.[17–21] Some of these compounds have been used successfully in pharmacological studies suppressing T-cell proliferation by DPIV-inhibition[20,21] and inhibiting mice brain PEP activity.[22,23]

Since pyrrolidides and thiazolidides lack the carboxylic group of proline, they bind only to the S-subsites of the target peptidases. Therefore, C-terminal elongation of the inhibitor structure to provide productive interactions with the S′-subsides of the binding site is not possible.

Thus we have been looking for compounds binding to proline-specific enzymes at both binding sites and discriminating others as well as being stable enough to perform biological investigations. E. Shaw introduced peptidyl sulphonium methyl ketones as inhibitors of cysteine proteases,[24] exploring possible interactions between the positive charged inhibitor molecule and the high nucleophilic potential of active site thiolate of the target enzymes. Since it was found that especially prolyl endopeptidases posses a highly reactive active site nucleophile[25,26] we adopted Shaw's strategy and tested ammonium (and pyridinium) methyl ketones as inhibitors of proline-specific enzymes. Since proline-containing compounds tend to form ring structures intra-molecularly, we selected the tetra coordinated ammonium derivatives to additionally hinder this tendency.

MATERIALS AND METHODS

General Synthesis and Product Analysis

All reagents for synthesis were purchased from commercial sources and solvents were dried using common procedures. HPLC-analysis of all compounds were performed using a Merck-Hitachi-system equipped with a photodiode array detector. FPLC-filtration of enzyme-inhibitor solutions were done on a Pharmacia-FPLC-system. ^{13}C-NMR spectra were recorded on a Bruker WP-200 spectrometer. Chemical shifts (δ) are reported in parts per million relative to an internal tetramethylsilan standard in case of $CDCl_3$ as solvent and relative to dioxan (Merck, Germany), in case of the D_2O-solutions. Mass spectra were taken in positive ion mode on a Finnigan Mat 90 double focusing instrument (Bremen, Germany), operating at a 5 kV

accelerating voltage. Xenon was a source of the primary beam (Ion Tech saddle field gun), adjusted to 7 kV and 2 mA. Samples were dissolved in methanol, mixed with an equal amount of glycerol matrix and approximately 1.0 nM of substance was analyzed using magnetic scan at a rate of 5 s/decade. Data were acquired and processed by a PDP-11 minicomputer system.

Synthesis of the Inhibitors

Peptidyl chloromethyl ketones as starting material in this work were obtained according to known standard procedures as previously described.[27,28] The ammonium methyl ketones were synthesized by treatment of the corresponding peptidyl chloromethyl ketones with the appropriate base in acetone for 3 days at room temperature. The products were obtained as oils. Freeze-drying resulted in lyophylized powders. Purity of the intermediate products was checked by HPLC. Structural integrity was confirmed by ^{13}C-NMR and mass spectroscopy. Due to the extreme hygroscopicity of the final ammonium methyl ketones insufficiently exact CHN-data could be obtained. HPLC-analysis of all compounds were performed using a Merck-Hitachi-system equipped with a photodiode array detector. The molecular weight and composition was confirmed by FAB-mass spectroscopy (Figure 1).

Z-Phe-Ala-CH_2-$N^+(CH_3)_3/Cl^-$, MW: 460.99

2.3 g Z-Phe-Ala-chloromethylketone (5.7 mM) were dissolved in 10 ml dry acetone and kept at −40°C. To this solution was added 1.0 ml (11.9 mM) of dry trimethylamine at −40°C. The reaction vessel was hermetically closed and left at room temperature. After 3 days solvent and non-reacting base were evaporated, and the residue dissolved in chloroform/methanol (9:1, v/v) and separated from unconverted starting material at a silical gel 60 column using pure methanol for elution. Rechromatography at Sephadex G 10 in H_2O and Sephadex LH 20 in methanol gave a white, pure but hygroscopic product which did not crystallize and was lyophilized for storage. The product is freely water soluble. TLC on silica gel coated aluminium plates gave a Rf of 0.61 when developed using n-butanol, H_2O, glacial acetic acid, ethyl acetate (1/1/1/1). Yield: 1.2 g (2.6 mM), 45.6%.

^{13}C NMR (50.327 MHz, $CDCl_3$) δ 201.20 (-CH_2-**C**O-), 172.67 (-NH-**C**O-), 156.42, (-O-**C**O-), 136.52, 136.27, 129.29, 127.85 (-CH-CH_2-**C**$_6H_5$), 136.42, 128.44, 127.23, 126.85, (-CO-O-CH_2-**C**$_6H_5$), 67.07 (-**C**H_2-O-CO-), 66.49 ($^+$(**C**H_3)$_3$N-**C**H_2-), 56.31 (-O-CO-NH-**C**H-), 54.53 (**C**H_3-CH-), 53.88 ($^+$(**C**H_3)$_3$N-), 37.62 (-O-CO-NH-CH-**C**H_2-), 15.24 (**C**H_3-CH-); FAB-MS $(M^+H)^+$ 426.2;

The other substances were obtained as described above as non-crystallizable, hygroscopic, freeze dried powders, in up to 50% yield.

Z-Ala-Phe-CH_2-$N^+(CH_3)_3/Cl^-$, MW: 460.99

^{13}C NMR (50.327 MHz, D_2O) δ 200.92 (-CH_2-**C**O-), 176.71 (-NH-**C**O-), 158.01 (-O-**C**O-), 136.16, 128.62, 127.96, 127.63, (-CH-CH_2-**C**$_6H_5$), 136.61, 129.53, 129.19, 127.96 (-CO-O-CH_2-**C**$_6H_5$), 67.91 (-**C**H_2-O-CO-), 67.18 ($^+$(**C**H_3)$_3$N-**C**H_2-), 59.15 (-CO-NH-**C**H-CH_2-), 51.27 (**C**H_3-CH-), 54.14 ($^+$(**C**H_3)$_3$N-), 34.63 (-O-CO-NH-CH-**C**H_2-), 16.62 (**C**H_3-CH-); FAB-MS $(M^+H)^+$ 426.3;

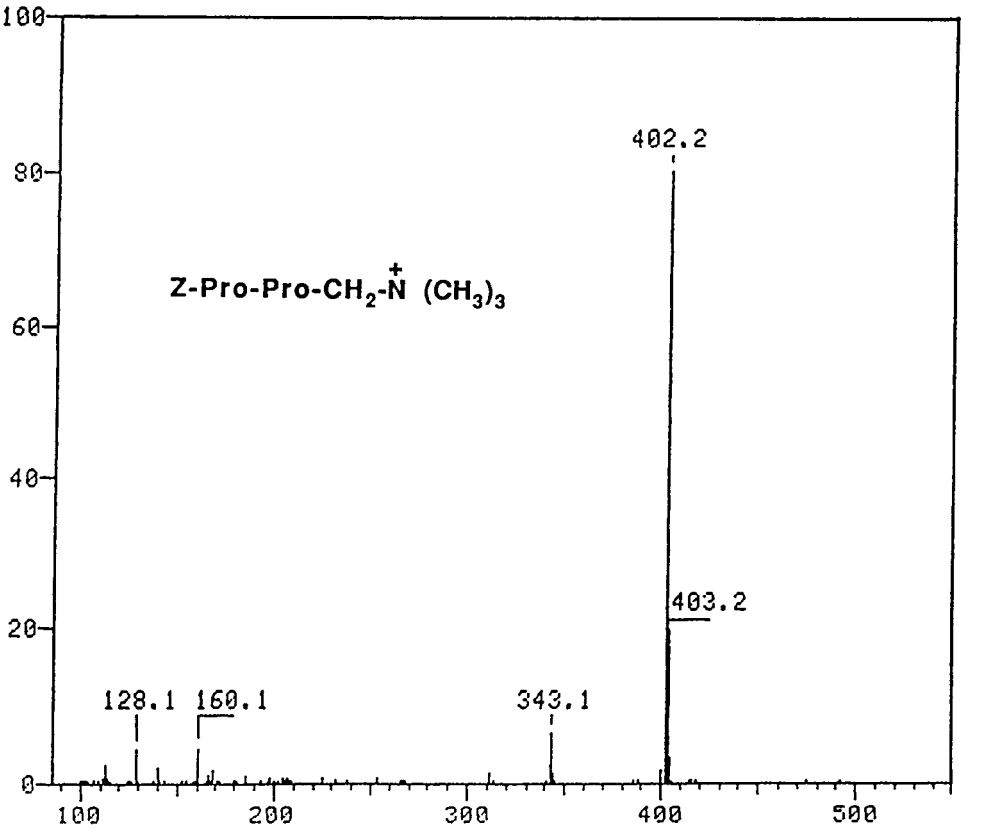


Figure 1 Mass spectrum of Z-Pro-Pro-(trimethyl) ammonium methyl ketone, taken in positive ion mode on a Finnigan Mat 90 double focusing instrument (Bremen, Germany), operating at a 5 kV accelerating voltage. Xenon was a source of the primary beam (Ion Tech saddle field gun), adjusted to 7 kV and 2 mA. Samples were dissolved in methanol, mixed with an equal amount of glycerol matrix and approximately 1.0 nM of substance was analyzed using magnetic scan at a rate of 5 s/decade. Data were acquired and processed by a PDP-11 minicomputer system. Y-axis; Relative intensity (%). X-axis: m/z.

Boc-Phe-Pro-CH_2-$N^+(CH_3)_3/Cl^-$, MW: 454.01
^{13}C NMR (50.327 MHz, D_2O) δ 201.35 (-CH_2-CO-), 172.64 (-N-CO-), 157.03 (-O-CO-), 136.60, 129.71, 129.12, 127.70 (-C_6H_5), 81.42 ($(CH_3)_3$C-O-), 63.91 ($^+(CH_3)_3$N-CH_2-), 55.31 (-CO-NH-CH-CH_2-), 55.25 ($^+(CH_3)_3$N-), 55.23 (-N(CH_2-CH_2-CH_2-CH_2)CO-), 47.89 (-N(CH_2-CH_2-CH_2-CH_2)CO-), 37.46 (-NH-CH-CH_2-), 29.18 (-N(CH_2-CH_2-CH_2-CH_2)CO-), 28.07 ($(CH_3)_3$C-), 25.09 (-N(CH_2-CH_2-CH_2-CH_2)CO-); FAB-MS $(M^+H)^+$ 418.3;

Z-Pro-Pro-CH_2-$N^+(CH_3)_3/Cl^-$, MW: 437.95
^{13}C NMR (50.327 MHz, $CDCl_3$) δ 201.89 (-CH_2-CO-), 171.72 (-N-CO-), 155.03 (-O-CO-), 136.98, 128.60, 127.87, 126.68 (-C_6H_5), 66.55 (-CH_2-O-CO-), 64.90 ($^+(CH_3)_3$N-CH_2-), 53.52 ($^+(CH_3)_3$N-), 58.42 (-N(CH_2-CH_2-CH_2CH_2)CO-), 47.08,

44.76 (-N(CH_2-CH_2-CH_2-CH_2)CO-), 28.98, 27.53 (-N(CH_2-CH_2-CH_2-CH_2)CO-), 25.85, 24.55 (-N(CH_2-CH_2-CH_2-CH_2)CO-); FAB-MS $(M^+H)^+$ 402.2;

Z-Pro-Pro-CH_2-$N^+C_5H_5/Cl^-$, MW: 457.96

^{13}C NMR (50.327 MHz, D_2O) δ 201.31 (-CH_2-CO-), 173.59 (-N-CO-), 156.16 (-O-CO-), 147.21, 145.80, 136.20 ($C_5H_5N^+$), 136.73, 129.13, 128.61, 127.95 (-C_6H_5), 66.50 (-CH_2-O-CO-), 63.66 ($^+(CH_3)_3N$-CH_2-), 58.73, 58.20 (-N(CH_2-CH_2-CH_2-CH_2)CO-), 47.64 (-N(CH_2-CH_2-CH_2-CH_2)CO-), 29.98, 28.14 (-N(CH_2-CH_2-CH_2-CH_2)CO-), 25.05, 23.90 (-N(CH_2-CH_2-CH_2-CH_2)CO-); FAB-MS $(M^+H)^+$ 422.1;

HBr/H-Pro-Pro-$CH_2N^+(CH_3)_3/Br^-$ was obtained by treatment of Z-Pro-Pro-$CH_2N^+(CH_3)_3/Cl^-$ with 33% HBr/glacial acetic acid for 30 min and precipitation of the white product using diethyl ether. HCl/H-Phe-Pro-$CH_2N^+(CH_3)_3/Cl^-$ was obtained by deprotection of Boc-Phe-Pro-$CH_2N^+(CH_3)_3/Cl^-$ using 1.1 n HCl in glacial aceitc acid for 30 min and precipitation of the white product using diethyl ether. Because of the hygroscopicity and instability of both compounds, NMR and mass spectroscopic data are not available.

Kinetic Experiments

Kinetic experiments were performed using a Carl-Zeiss-Jena UV-Vis spectrometer M40 equipped with electrical heat and temperature controlled cuvette holders. The instruments was set up with a ROM-cartridge allowing on-line calculations of zero-order, first-order-rate constants from raw data as extensively described previously.[29]

Enzymes

Bacterial prolyl endopeptidase from *Flavobacterium meningosepticum* was purchased as a lyophilized powder from Miles (USA) or kindly provided as recombinant protein from *Escherichia coli* by Dr. T. Diefenthal (Weissheimer Research, Germany) exhibiting a specific activity (at 30°C and pH 7.6 using Gly-Pro-Pro-pNa as substrate) of 14 U/mg and 77 U/mg, respectively. Dipeptidyl peptidase IV was prepared as described previously[14] having a specific activity of 33 U/mg at 30°C and pH 7.6 using Gly-Pro-pNA as substrate. The substrates were synthesized as previously described.[30] Reversibility analysis was performed using a 15 × 55 mm column, filled with BIORAD Bio-Gel P-2. Typically, 0.4 ml solution containing enzyme, inhibitor and 0.04 M sodium phosphate buffer, ionic strength of 0.125, pH 7.6 after one hour's incubation were separated on the column. The protein containing fractions were combined and the residual activity of the enzyme estimated.

Stability of the Inhibitors

The compounds were incubated in test buffer and their inhibitory activity checked by withdrawing aliquots and collecting inhibition data as described. All three Z-protected peptidyl ammonium methyl ketones are 100% stable for more than 30 h under the condition used. In contrast, the deblocked HBr/H-Pro-Pro-$CH_2N^+(CH_3)_3/Br^-$ decomposed rather quickly. The inhibitor lost its activity (measured by initial rate estimation) with a half-life of 15.1 min. This corresponds to an increase in the

UV-absorption of the inhibitor solution as 310 nm, probably due to pyrazin formation. the half-life of this process was calculated to be 14.7 min.

RESULTS AND DISCUSSION

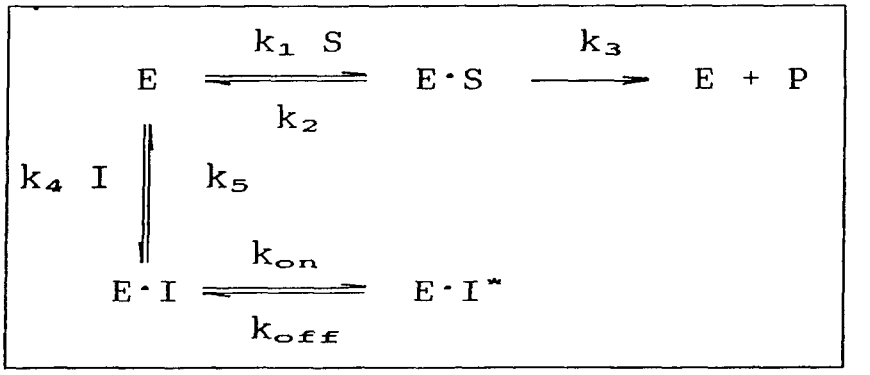
Incubation of PEP and DP IV with substrate analogous peptidyl ammonium methyl and pyridinium methyl ketones in presence of substrate[29] resulted in the formation of non-covalent inhibitor complexes (E·I and E·I* in Scheme 1). Progress curves (Figure 2) of the inhibition of PEP and DP IV by peptidyl ammonium methyl ketones have been analyzed according to a slow-binding-inhibition mechanism[31] and the kinetic parameters arre given in Table 1. PEP and Z-Pro-Pro-$CH_2N^+C_5H_5$ form an E·I* complex with an apparent second order rate constant of $2.37 \times 10^5\ M^{-1}\ s^{-1}$. Figure 3 shows the dependence of the rate of the formation of the E·I*-complex on the inhibitor concentration, demonstrating the slow-binding mechanism.

In contrast Boc-Phe-Pro-$CH_2N^+(CH_3)_3/Cl^-$ does not exhibit slow-tight binding inhibition of PEP but reacts by a "classical" competitive inhibition mechanism with the target enzyme resulting in a K_i-value of 120 nM (Table 1).

Incubating the enzymes tested for longer time periods (up to 12 h) showed that no inactivation could be observed as in the case of cysteine proteases[24] reacting irreverisbly with peptidyl sulphonium methyl ketones.

Table 1 Kinetic parameters of the inhibition of prolyl endopeptidase by peptidyl ammonium methyl ketones in 40 mM sodium phosphate buffer, $\mu = 0.125$ at pH 7.6 and 30°C

Inhibitor	K_i (nM)	K_i^* (nM)	k_{on} (s^{-1})
Z-Pro-Pro-$CH_2N^+C_5H_5/Cl^-$	9.3	1.8	0.0022
Boc-Phe-Pro-$CH_2N^+(CH_3)_3/Cl^-$	120.0	—	—
Z-Pro-Pro-$CH_2N^+(CH_3)_3/Cl^-$	710.0	64.0	0.0062
Z-Phe-Ala-$CH_2N^+(CH_3)_3/Cl^-$	75000.0	16000.0	0.0019
Z-Ala-Phe-$CH_2N^+(CH_3)_3/Cl^-$	100000.0	—	—



Scheme 1 Functional scheme of slow-binding inhibition according to Morrison.[31]

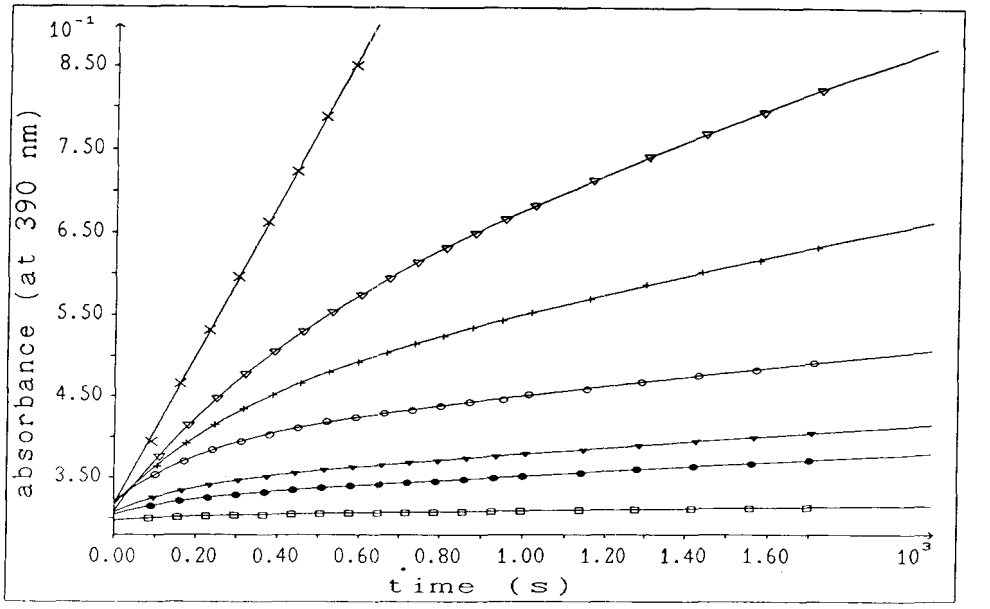


Figure 2 Progress curves for the generation of 4-nitroaniline by PEP-catalyzed hydrolysis of Gly-Pro-Pro-4-nitroanilide in the presence of Z-Pro-Pro-$CH_2N^+(CH_3)_3/Cl^-$. Substrate concentration 0.53 mM. Inhibitor concentrations: (X) 0.0, (▽) 1.2, (+) 2.1, (○) 4.2, (▼) 8.4, (●) 12, (□) 25 μM. PEP was 17 nM. Measurements in 40 mM sodium phosphate buffer, $\mu = 0.125$, 30°C.

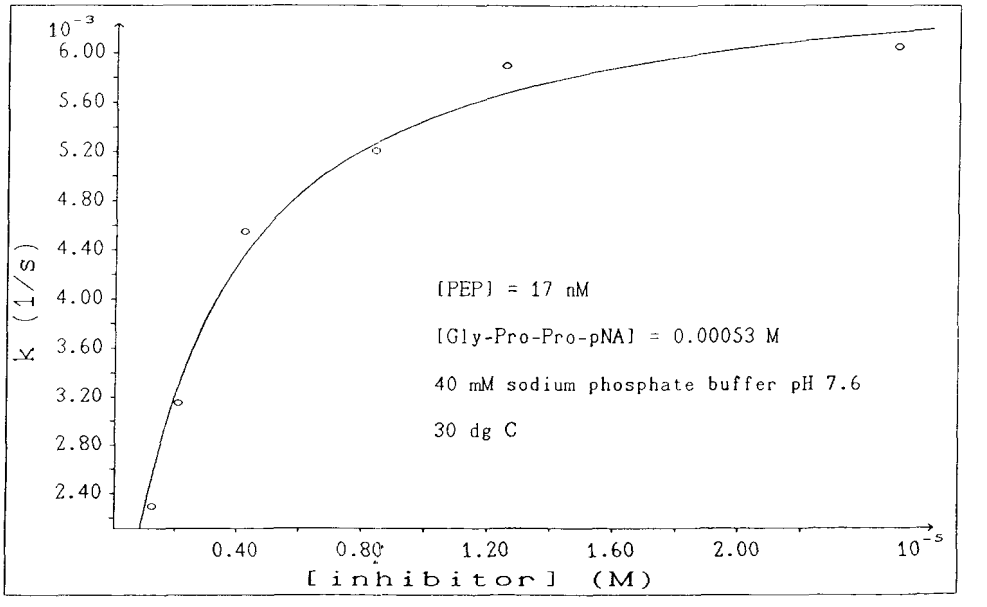


Figure 3 Dependence of the apparent first order rate constant k for the establishment of the equilibrium between E·I and E·I*, during inhibition of PEP by Z-Pro-Pro-$CH_2N^+(CH_3)_3/Cl^-$, measurements in presence of substrate, Gly-Pro-Pro-4-nitroanilide 0.53 mM, PEP = 17 nM, 40 mM sodium phosphate buffer, pH 7.6, $\mu = 0.125$, 30°C.

While the N-terminal protected inhibitors are absolutely stable and keep their inhibitory power for more than 30 h after incubation in phosphate buffer at pH 7.6 and 30°C, the N-terminal deprotected compounds decompose rapidly. H-Pro-Pro-$CH_2N^+(CH_3)_3$ has a half-life of only 15 min under this conditions. Similar instability of N-terminal deprotected dipeptidyl methyl ketones was described previously suggesting cyclization to pyrazin derivatives.[28] Thus, only the equilibrium constant K_i for the establishment of the initial E·I complex and the apparent second order rate constant of the formation of the secondary EHI* complex between DP IV and inhibitor could be determined. H-Pro-Pro-$CH_2N^+(CH_3)_3$ inhibits DP IV with a K_i of 9.3 μM and an apparent second order rate of k_{on}/K_i of 2713 $M^{-1}\,s^{-1}$. The newly synthesized structures are among the most potent reversible inhibitors for PEP and DP IV and are interacting with both enzymes by a slow-binding mechanism.[31]

In contrast, the substrate analog of the chymotrypsin-like proteases, Z-Ala-Phe-$CH_2N^+(CH_3)_3$, does not inhibit chymotrypsin, subtilisin and the subtilisin-like serine protease thermitase at all. Only weak competitive inhibition of papain ($K_i \cong 3$ mM) by Z-Phe-Ala-$CH_2N^+(CH_3)_3$ could be observed.

Because of this discrimination for other proteases, the easy preparation of the compounds, their stability if N-terminal protected and the high water solubility of the salts, investigations to improve the stability of the deprotected derivatives by introduction of bulkier trialkyl ammonium residues and the application of the new structures in biological systems are in progress.

A similar inhibitory principle has recently been introduced by Janda and coworkers in approaching the inhibition of HIV-protease by phosphonyl ammonium methyl-ketones.[32] So, our new group of compounds could provide an alternate pathway to appropriate protease inhibitors.[33]

Acknowledgement

This work was supported in part by the German *Bundeministerium für Forschung und Technologie* Grant 03 10259 A (to H.-U. D.) and by the Swedish *Board for Technical Development (NUTEK)* Grant DNR 9003385 (to J.S.).

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